AD_____
(Leave blank)

Award Number: W81XWH-07-1-0393

TITLE:
Biological and Computational Modeling of Mammographic Density and Stromal Patterning

PRINCIPAL INVESTIGATOR:
Victoria Seewaldt, M.D.

CONTRACTING ORGANIZATION:
Duke University
Durham, North Carolina 27710

REPORT DATE: July 2010

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2010	Final	01 July 2007 to 30 Jun 2010

4. TITLE AND SUBTITLE
Biological and Computational Modeling of Mammographic Density and Stromal Patterning

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0393

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Victoria Seewaldt, M.D.
Email: seewa001@mc.duke.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University, Room 221A MSRB, Durham, NC 27710

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Ft. Detrick, MD 21702

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Here we have worked to correlate computational models of mammographic and stromal patterning with clinical outcome leading to the construction of multi-disciplinary tools for the classification of breast cancer risk and response to prevention strategies. To this end we have currently evaluated mammographic density in 75 women taking tamoxifen chemoprevention and 75 high-risk women who elected not to take tamoxifen using pattern analysis of 1) serial mammograms, 2) serial breast Magnetic Resonance Imaging, and 3) Random Periareolar Fine Needle Aspiration (RPFNA). We observe no correlation between the presence or absence of atypia after tamoxifen prevention and changes in mammographic density. Two women developed breast cancer while taking tamoxifen who had a progressive decrease in mammographic density. These findings demonstrate the viability of using RPFNA to assess prevention response.

15. SUBJECT TERMS
Mammographic density, breast cancer risk, random periareolar fine needle aspiration, early detection.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE		10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction……..…..4

Body……..4-6

Key Research Accomplishments…….……..7

Reportable Outcomes…… 7 - 8

Conclusion…… 8

References…….8-9

Appendices……10

INTRODUCTION:

Mammographic density serves as independent marker of short term breast cancer risk and a surrogate marker of response to a variety of prev ention agents (1-3). Although a m ajority of breast cancers are ep ithelial in origin, there is evidence that stromal content of the breast is an important predictor or mammographic density. There is increasing evidence that the s troma plays a role in brea st cancer initiation (4). However, cu rrently we lack an understanding of how mammographic density is affected by the individual contribution of epithelial and stromal components and the biological potential of strom al and/or epithelial cells. The goals of this synergistic grant proposal are to develop com putational and biologica l tools to investigate the relationship betwee n mammographic density, strom al content of the breast, a nd the role of strom al/epithelial intera ctions in regulating proliferation, and ultimately, short-term breast cancer risk. To ach ieve these goals we bring together investigators with expe rtise in m athematical f ractal pattern assessm ent, 3-D m odels of strom al/epithelial interactions, and c linical breast cancer risk assessm ent. Together we propose to correlate com putational models of mammographic and strom al patterning with biological assays of stromal/epithelial proliferation, and clinical outcome lea ding to the construc tion of m ulti-disciplinary tools for the c lassification of breast c ancer risk a nd response to prevention strategies.

Random Periareolar Fine Needle Aspiration (RPFNA) is a research technique that h as been prospectively validated to assess *1)* short-term breast can cer risk and *2)* response to chem oprevention in high-risk wom en (5-7). W hile RPFNA was orig inally develope d to evaluate early epith elial chang es, RPFNA also provides a repres entative sam pling of strom al cells in high-risk wom en. prevention agents, and therefore, mammographic In this S ynergy Proposal, we are currently testing the ***hypothesis*** that in wom en with epithelial atypia, 1) mammographic and strom al pa tterning does no t consistently predict the degree of e pithelial a typia (measured by Masood Cytology Index) and 2) m ammographic density m ay not be a reliable m easure of epithelial response to prevention agents.

BODY:

Objective 1: To investigate the relationships between mammographic density, mammary stromal patterns and computational image analysis of the breast. The goals of this aim are to 1) Quantitate the stromal-epithelial cell ratios obtained from RPFNA and quantitate imaged breast density computer modeling; 2) Perform comparisons and correlations between RPFN A strom al-epithelial cell r atios, and m ammographic density; 3) Statistically examine the relation ship between mammographic density, MRI fibroglandular volume, and RPFNA stromal to epithelial composition and stromal patterning.

Task 1: RPFNA, Digitizing, Annotation, and Posting.

TIMELINE: ***Years 1-2:*** 50 RPFNA will be perf ormed in high-risk wom en, slides will be prepar ed, cytology assessed, slides will be digitized, annotated and posted.

MILESTONES: ***Year 1:*** 50 RPFNA performed, tested, and posted.

RPFNA is a research technique that has been prospectively validated to assess *1)* short-term breast can cer risk and *2)* response to chemoprevention in high-risk wome (5-7). RPFNA cytology is assessed by Masood score by a single dedicated pathologist (***Carola Zalles***) who has >10 years experience in assessing RPFNA cytology (5-7). This allows for reproducible id entification of early cy tological changes in mammary epithelial cells. Epithelial and stromal cells are counted in 4 individuals RPFNA slides.

Cellular morphology	Cellular pleomorphism	Myoepithelial cells	Aniso-nucleosis	Nucleoi	Chromatin clumping	Score
Monolayer	Absent	Many	Absent	Absent	Absent	**1**
Nucl. overlap	Mild	Moderate	Mild	Micro-nucleoli	Rare	**2**
Clustering	Moderate	Few	Moderate	Micro-nucleoli	Occasional	**3**
Loss cohesion	Conspicuous	Absent	Frequent	Macro-nucleoli	Frequent	**4**

We performed serial RPFNA on 75 high-risk controls and 75 high-risk women taking tam oxifen chemoprevention. Women not taking tam oxifen were risk-matched to the 75 wom en who took t amoxifen. Each wom an underwent an

average of 3 RPFNA. A total of 403 RPFNA were analyzed. Subject demographics are presented in **Table 1**. All 403 RPFNA slides have been digitized, annotated, on a password protected server.

The average time of total observation for women was 15 months (range 12 to 54 months) and the average time on tamoxifen prevention was 14 months (range 12 to 50 months). The average age of women was 42 (range 39 to 52). Seventy percent of women were premenopausal and 30% were either perimenopaual or postmenopausal. Twenty-two percent of women were African American and 78% were Caucasian. See **Table 1** for subject demographics.

We previously used RPFNA to test for cytological response to tamoxifen chemoprevention in high-risk women with atypia. We observed that disappearance of atypia occurs within the first 12 months of initiating tamoxifen. After 12 months, women do not have disappearance of atypia. In the 75 women taking tamoxifen chemoprevention in this study, we observe that 32/75 women have disappearance of atypia after 12 months tamoxifen prevention and 43 women have persistent atypia. This is consistent with the Breast Cancer Prevention Trial (P1) which demonstrated a 50% reduction in estrogen receptor-positive breast cancer.

Task 2: Analysis of Epithelial/Stromal Counts.

TIMELINE: ***Years 1-2: Cytological Quantization:*** Using a standard volume of suspended RPFNA cells, four cytology slides will be generated. Epithelial cell and stromal cell counts will be quantitated by a blinded cytologist in triplicate.

***Computational Pattern Analysis*:** Fractal pattern analysis of epithelial and stromal cells will be performed on digitized images of fixed cell slides from the RPFNA.

MILESTONES: ***Year 1:*** Stromal and Epithelial Cell Counts will be tested from 50 subjects using cytological quantitation, biochemical and computational pattern analysis.

Epithelial/Stromal Counts: We performed epithelial cell counts on a standard volume of RPFNA cells from 403 RPFNA slides from 150 subjects described above in **Task 1**. Total cell counts are determined from all RPFNA slides. Stromal cell counts and computational analysis is on-going. We observed a correlation between a decrease in cell counts and the presence or absence of atypia after 12 months tamoxifen chemoprevention ($p<0.001$). Of the 32 women who had disappearance of atypia all had >75% decrease in RPFNA cell counts. For the 43 women who had persistent atypia, no subject had a >25% decrease in cell counts after 12 months tamoxifen prevention.

Task 3: Analysis of Mammographic Density:

TIMELINE: ***Year 1-2:*** Mammographic density will be assessed quantitatively using 1) visual assessment of mammographic density and 2) a novel automated computer method

MILESTONES: ***Year 2:*** 100 Mammograms analyzed by visual assessment and computer automated methods. A total of 250 (150 old; 100 new) will be completed.

Mammographic Density: Over 559 serial screen-film mammograms were digitized from the 150 women described in **Task 1**: 75 high-risk women taking tamoxifen prevention and 75 high-risk women who elected not to take tamoxifen. Woman had an average of 3 mammographic determinations. Mammograms from both breasts were digitized, including cranial caudal and medial lateral views.

Over 559 serial screen-film mammograms were digitized from 75 women taking tamoxifen prevention and 75 controls using a new Howtek MultiRad 860 digitizer. The anonymized mammographic images were stored on our private computer network and referenced in the dat abase. Mammographic density was assessed quantitatively using established computer modeling techniques. We are using the public Digital Database for Screening Mammography. To verify the reproducibility and robustness with respect to imaging technique, 5 mammographic density we compared the medio-lateral oblique and craniocaudal views of the same digitized breast.

We find in the course of this analysis that assessment of mammographic breast density by analysis of films suffers from variability. In order to effectively and consistently analyze mammographic density, we are currently using breast MRI to assess breast density.

Task 4: Analysis of MRI

TIMELINE: ***Year 1-2:*** MRI slices will be segmented manually and total voxel volumes for the fibroglandular tissue will be computed over the whole breast. Patterns of suspicious MRI signal enhancement will be preliminarily evaluated.

MILESTONES: ***Year 2:*** Analysis of 100 MRIs will be completed. A total of 250 (150 old; 100 new) will be completed.

MRI Image Analysis. MRI differentiates fatty and fibroglandular tissue with high precision and accuracy, therefore allowing a different assessment breast density (8,9). We performed and collected an average of 3.1 breast MRI on each of our 150 subjects that are described in **Task 1**. All MRI were performed with a commercial system using a dedicated breast coil. The digital files were obtained from the Picture Archiving and Communication System (PACS) and placed on our private computer network for the specified analysis. We are currently performing a preliminary semi-automatic analysis of the 3-D MRI images. MRI slices are segmented manually and total voxel volumes for the fibroglandular tissue are computed over the whole breast. Breast MRI detected 5 breast cancers in the 150 subjects described in **Task 1**. All four subjects had a decrease in mammographic density.

Task 5: Statistical analysis

TIMELINE: ***Years 1-2:*** Statistical analysis will be performed to correlate mammographic density with, MRI patterning, stromal cell counts, and stromal patterning.

MILESTONES: ***Year 2:*** Statistical analysis will be completed.

Statistical Analysis: Statistical comparison are on-going and methods include, *1)* Pearson's correlation coefficient, *2)* Spearman rank correlation coefficient, and *3)* mutual information. *Pearson's correlation coefficient* measures linear dependence between random variables. *Spearman rank correlation coefficient* (10) can show correlation between rank-ordered data. Since the data is ranked, *1)* the values are not used directly; *2)* the measure of correlation is independent of scales; *3)* no assumptions are made about the distribution of the underlying data. *Mutual information* (11) is a method for measuring the general statistical dependence between random variables. Mutual information will be computed to test whether a more general statistical dependence exists between mammographic density, fractal patterning, and stromal/epithelial counts. Questions that we are currently testing include:

a) Do stromal and/or epithelial counts predict mammographic density? We predict that stromal cell counts and the stromal/epithelial ratio will be the primary predictor of mammographic density.

Observation to date: We have completed this analysis for 150 subjects and observe that epithelial counts do not predict mammographic density. We observe a direct correlation between epithelial cell counts and Masood Cytology abnormalities ($p<0.001$).

b) Is there a correlation between the presence or absence of atypia after tamoxifen chemoprevention and changes in mammographic density? We predict that there will not be a correlation.

Observation to date: We tested for a correlation in the 75 subjects described in **Task 1** who took tamoxifen chemoprevention. There was no correlation ($p>0.5$) when the data analysis was performed for individual women or individual breasts. Two women developed breast cancer while taking tamoxifen chemoprevention. Both women had a decline in mammographic density. In contrast, a minority of women had correlation between mammographic density and disappearance of atypia in RPFNA.

c) Is there a correlation between mammographic density, mammographic and stromal fractal patterning, and RPFNA Masood epithelial cytology? We predict that in subjects with hyperplasia (vs. non-proliferative cytology) there will be a direct relationship, however, in subjects with atypia there will not be a direct correlation. These studies will provide rational for developing multi-modality measures of short-term breast cancer risk and response to prevention strategies.

Observation: There is not direct correlation.

OBJECTIVE 2: **To test whether increased mammographic density correlates with increased stromal proliferation.** To accomplish this aim we are using combinations of 1) defined epithelial cell and 2) patient-derived epithelial cells obtained RPFNA will be co-cultured with stroma isolated from subjects with high- and normal-

mammographic density. Co-culture methods will include 3-D culture and 3-D rotary bioreactor culture, stromal and epithelial cells will be tested for proliferation and transcriptional activation.

Task 1: Isolation of Mammary Stromal and Epithelial Cells from RPFNA

TIMELINE: ***Years 1-2:*** Obtain matched HMECs and stromal cells from high-risk patients with high and low-medium mammographic density.

MILESTONES: ***Year 2:*** Obtain 10 matched sets of stroma and epithelial cells from RPFNA.

Observations to date: We have collected matched HMECs and stroma from 10 high-risk patients with high mammographic density. Three immortalized lines were derived.

Task 2: Epithelial/Stromal Co-Culture.

TIMELINE: ***Years 1-2:*** Perform 3-D culture with combinations of stroma and epithelial cells obtained from women with high and low-medium mammographic density.

MILESTONES: ***Year 1:*** 3-D culture performed on 5-10 samples.

We performed co-culture of defined and patient-derived epithelial/stroma cells. Cells have been isolated from high-risk women with *1)* atypia who have *2)* high or normal mammographic density. Co-culture methods include 3-D bioreactor culture. We tested for dominance of stroma versus epithelium. In the studies we performed we did not observe dominance of stroma over epithelium or vica verca. These observations lead us to believe that co-culture is not adequate for maintaining the breast microenvironment adequately. Our findings are disappointing but important because they have lead to new studies. As a result we are now performing direct microdissection and testing of protein signaling in mammary epithelial and stromal cells directly obtained from a woman's breast.

KEY RESEARCH ACCOMPLISHMENTS:

1) RPFNA is a viable means to track response to chemoprevention in high-risk women with mammary atypia.

2) Disappearance or persistence of atypia in RPFNA cytology in women taking 12 months of tamoxifen prevention does not correlate with changes in mammographic density.

3) Breast Magnetic Resonance Imaging appears to be a more reliable measure of breast density than film determination of mammographic density.

REPORTABLE OUTCOMES:

Manuscripts

Baker, J., Zalles, Seewaldt, V.L. Methylation of the estrogen receptor alpha promoter (*ESR1*) in RPFNA does not predict response to Tam prevention. *Cancer Epi Biomarker Prev.* 17:11-27, 2008. (PMCID18708376).

Ibarra, C., Wilke, L., Yee, L., Kulkarni, S., Wood, M., Garber, J., Stouder, A., Grant, T., Broadwater, G., and Seewaldt, V.L. Random Periareolar Fine Needle Aspiration is highly reproducible in a cooperative multi-institutional trial. *Cancer Epi Biomarkers Prev*, 18:1379-1384, 2009 (PMCID19258476).

Lo, J, Barron, A, and Seewaldt, VL. Presence or absence of atypia in RPFNA does not correlate with mammographic density changes after 12 months tamoxifen prevention. Submitted Cancer Epi Biomarkers and Prevention, 2010.

Presentations

1. Lo, J, Barron, A, and Seewaldt, VL. Presence or absence of atypia in RPFNA does not correlate with mammographic density changes after 12 months tamoxifen prevention. Presented *Era of Hope*, June 2008.

2. Seewaldt, V.L. Modeling Breast Cancer Risk. Plenary Talk, Breast SPORE, Washington, DC, 2010.
3. Seewaldt, V.L. Measurement of mammographic density in high-risk women before and after tamoxfien. Plenary Talk, AACR Prevention, Phil1adelphia, PA, 2010.

Career Development

1. Nicholas D'Amato, PhD candidate.
2. Catherine Ibarra, Komen Career Catalyst, 2010.
2. Stacy Millon, PhD, 2010.
3. Molly Gregas, PhD, 2010
4. Julie Ostrander, K07 Award, 2010

Funding

1. Victoria Seewaldt, NIH/NCI 1R01CA155664-01, funded 07/2010.
2. Dihua Yu, Victoria Seewaldt, KG091020, funded 12/2009.
3. Julie Ostrander, K07, funded 08/2010.
4. Victoria Seewaldt, R01 RFA Biology of Premalignancy, not funded.

CONCLUSIONS:

a) RPFNA epithelial counts do not predict mammographic density. Stromal cell counts and the stromal/epithelial ratio appear to be the primary predictor of mammographic density.
b) Persistence or absence of atypia in RPFNA cytology after 12 months tamoxifen prevention do not predict changes in mammographic density.

.

"So what":

To our knowledge, this is the first study to attempt to develop a computational model of mammographic density and correlate this model with stromal/epithelial biology. This project provides a rapid means to test for response to prevention and a new method to test for breast cancer risk. The innovative aspect of this work is that we are testing our observations in mammary stroma and epithelium directly isolated from high-risk women at the earliest stages of mammary carcinogenesis. These studies have created a unique interdisciplinary database of cytology, histology, genetic and cellular information for women at high-risk of developing breast cancer.

REFERENCES:

1. Wolfe, J.N., A.F. Saftlas, and M. Salane, *Mammographic parenchymal patterns and quantitative evaluation of mammographic densities: a case-control study.* American Journal of Roentgenology, 1987. 148(6): p. 1087-92.
2. Boyd, N.F., et al., *Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study.* Journal of the National Cancer Institute, 1995. 87(9): p. 670-5.

3. Cuzick, J., et al., *Tamoxifen and Breast Density in Women at Increased Risk of Breast Cancer.* J Natl Cancer Inst, 2004. 96(8): p. 621-628.
4. Alowami, S., et al., *Mammographic density is related to stroma and stromal proteoglycan expression.* Breast Cancer Research, 2003. 5(5).
5. Fabian, C.J., et al., *Short-term breast cancer prediction by random periareolar fine-needle aspiration cytology and the Gail risk model.* Journal of the National Cancer Institute, 2000. 92(15): p. 1217-27.
6. Fabian, C.J., et al., *A phase II breast cancer chemoprevention trial of oral alpha-difluoromethylornithine: breast tissue, imaging, and serum and urine biomarkers.* Clinical Cancer Research, 2002. 8(10): p. 3105-17.
7. Bean GR, Scott V, Yee L, Ratliff-Daniel B, Troch MM, Seo P, et al. Retinoic ac id receptor-beta2 promoter methylation in random periareolar fine needle aspi ration. Cancer Epidemiol Biomarkers Prev 2005. 14: p. 790-8.
8. Lee, N.A., et al., *Fatty and fibroglandular tissue volumes in the breasts of women 20-83 years old: comparison of X-ray mammography and computer-assisted MR imaging.[see comment].* AJR. American Journal of Roentgenology, 1997. 1 68: 501-6. 9. W ei, J., et al., *Correlation between mammographic density and volumetric fibroglandular tissue estimated on breast MR images.* Medical Physics, 2004. 31: 933-942.
10. Kendall, M. and J. Dickinson Gibbons, *Rank Correlation Methods*. 5th ed. 1990, London: Edward Arnold. 260.
11. Tourassi, G.D., et al., *Application of the mutual information criterion for feature selection in computer-aided diagnosis.* Medical Physics, 2001. 28: 2394-2402.
12. Pluim , J.P.W ., J.B.A. Ma intz, and M.A. Viergever, *Mutual-information-based registration of medical images: a survey.* IEEE Trans. Med. Imaging, 2003. 22: 986-1004.

APPENDICES:

None.

SUPPORTING DATA:

Table 1

High-risk subjects NOT on tamoxifen prevention	High-risk subjects on tamoxifen prevention	Controls not taking Tam	Total Subjects
Number	75	75	150
Average Age	43 (40-49)	42 (39-52)	42 (39-52)
Menopausal status Premenopausal Perimenopausal	 55/75 20/25	 59/75 16/25	 104/150 36/150
Race Caucasian African American	 60/75 15/75	 58/75 17/75	 118/150 26/150
Risk Atypia/LCIS DCIS	 19/75 6/75	 19/75 6/75	 19/150 6/150
Time of observation	14 mos (12 to 50 mos)	16 mos (12 to 54 mos)	15 mos (12 to 54 mos)
Duration of tamoxifen	14 mos (12 to 50 mos)	n/a	14 mos (12 to 50 mos)
Average density			
Average mammographic density	55% (range 30% to 55%)	52% (range 31% to 67%)	53% (range 30% to 67%)
Average number of RPFNA	3.1	3.3	3.2 (2-6 RPFNA)
RPFNA change Disappearance of atypia Persistence of atypia	 32/75 43/75	 2/75 73/75	 34/150 116/150
Development of breast cancer	2	3	5